(2)

CONTRACT NO: DAMD17-85-C-5077

TITLE: THE SCREENING AND EVALUATION OF EXPERIMENTAL ANTIPARASITIC DRUGS

PRINCIPAL INVESTIGATOR: DR. ARBA L. AGER, JR.

PI ADDRESS: UNIVERSITY OF MIAMI - School of Medicine -
CENTER FOR TROPICAL PARASITIC DISEASES
12500 S.W. 152nd. St.
Miami, Florida 33177

REPORT DATE: August 4, 1990

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick
Frederick, Maryland 21702-5012

ECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

a. REPORT SECURITY CLASSIFICATION
UNCLASSIFIED

1b. RESTRICTIVE MARKINGS

SECURITY CLASSIFICATION AUTHORITY

3. DISTRIBUTION/AVAILABILITY OF REPORT
Approved for public release; distribution unlimited

b. DECLASSIFICATION/DOWNGRADING SCHEDULE

. PERFORMING ORGANIZATION REPORT NUMBER(S)

5. MONITORING ORGANIZATION REPORT NUMBER(S)

a. NAME OF PERFORMING ORGANIZATION
University of Miami - CENTER OR TROPICAL PARASITIC DISEASES

6b. OFFICE SYMBOL (If applicable)

7a. NAME OF MONITORING ORGANIZATION

c. ADDRESS (City, State, and ZIP Code)
12500 S.W. 152nd St.
Miami, Florida 33177

7b. ADDRESS (City, State, and ZIP Code)

a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research and Development Command

8b. OFFICE SYMBOL (If applicable)

9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
DAMD17-85-C-5077

c. ADDRESS (City, State, and ZIP Code)
Fort Detrick
Frederick, Maryland 21702-5012

10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
62270A	3MI-62770A870	AJ	WUDA0F7580

1. TITLE (Include Security Classification)
(U) THE SCREENING AND EVALUATION OF EXPERIMENTAL ANTIPARASITIC DRUGS.

2. PERSONAL AUTHOR(S)
ARBA L. AGER, JR., Ph.D.

TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
ANNUAL	FROM 02/01/88 TO 01/31/89	1990 August	44

6. SUPPLEMENTARY NOTATION

7. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	13	
06	03	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Malaria, Plasmodium berghei, Plasmodium yoelii, Plasmodium, Drug-resistant malaria, Chloroquine-resistant malaria, Vitamin E, Antioxidant, African trypanosomiasis. (Over)

9. ABSTRACT (Continue on reverse if necessary and identify by block number) Malaria chemotherapeutic studies included a primary antimalarial blood schizontocidal test system (MM test) where 1502 compounds were evaluated against Plasmodium berghei with 168 exhibiting activity, and a secondary antimalarial program consisting of in depth evaluation of compounds against drug-sensitive and drug-resistant lines. Arteether in sesame oil was more active SC than PO. Chloroquine and primaquine were less toxic when given in fish oil. One sustained release formulation of qinghaosu was more active than another. Two stereoisomers (R and S) of a floxacrine analog interacted synergistically against malaria. Resistance to the R-stereoisomer developed slower than the S-stereoisomer or the racemate. A line resistant to qinghaosu was developed. Supllemental vitamin E did not alter the activity of several standard antimalarials. Changing the fatty acid profile in red blood cells by feeding plant and fish oils containing high levels of omega-3 polyunsaturated fatty rendered vitamin E-deficient mice cured of drug-sensitive, chloroquine or qinghaosu-resistant malaria.

A total of 360 compounds were tested for activity against drug-sensitive Trypanosoma (Over)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS

21. ABSTRACT SECURITY CLASSIFICATION
UNCLASSIFIED

. NAME OF RESPONSIBLE INDIVIDUAL
MRS. VIRGINIA M. MILLER

22b. TELEPHONE (Include Area Code)
(301)663-7325

22c. OFFICE SYMBOL
SGRD-RMI-S

D Form 1473, JUN 86 Previous editions are obsolete. SECURITY CLASSIFICATION OF THIS PAGE

18. SUBJECT TERMS (Cont.)

Trypanosoma rhodesiense, Drug-resistant trypanosomes, Pentamidine-resistant trypanosomes, Suramin-resistant trypanosomes, Melarsoprol-resistant trypanosomes, RAI.

19. ABSTRACT (Cont.)

rhodesiense. Activity was noted in 55 of these compounds. 91 active compounds were tested against lines resistant to either melarsoprol, pentamidine, or suramin.26 compounds were not cross resistant with either, of the 3 lines while 11 compounds were resistant to only one of the 3 resistant lines.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by U.S. Army.

_____Where copyrighted material is quoted, permission has been obtained to use such material.

_____Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

_____Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations.

_____In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use for Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources. National Research Council (NIH Publication No. 86-23, Revised 1985).

_____For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

_____In conduction research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

_____In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

_____In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Alba J Azer M.D. 8/4/90

PI - Signature Date

TABLE OF CONTENTS

TABLE OF CONTENTS (Cont.)

TABLE OF CONTENTS (Cont.)

INTRODUCTION

Malaria continues to be an extremely serious health problem to over 400 million people in the world each year. It causes tremendous morbidity with over 2 million annual fatalities. Control of malaria remains as elusive today as it did 5 years ago when several new vaccine prototypes were tested in man and optimistically projected to be used in man by 1990. The vaccine has not come to fruition and is in fact years away, if ever, from even attempting, to alleviate the malaria situation.

Vector control problems have continuously failed leaving malaria to be controlled by chemotherapy. Attempts to control **Plasmodium falciparum** and **Plasmodium vivax** via chemotherapy have encountered numerous obstacles. The foremost obstacle being multiple drug-resistance in **P. falciparum** and secondarily the toxicity of primaquine in its quest to control **P. vivax**. The only two new compounds emerging since the 1950's to combat the chloroquine resistant and often multiple drug-resistant **P. falciparum** are mefloquine and halofantrine. Resistance has been found already to both of these compounds and there are new toxicity problems emerging with mefloquine which may limit its usefulness.

Currently there is an urgent need for new antimalarial drugs to stop the increasing spread of multiple drug-resistant **P. falciparum** parasites and to block relapses found in **P. vivax**. In the quest to identify new active compounds we are currently testing 1,500 per year against drug-sensitive asexual blood stage induced malarial infections in the standard primary antimalarial test system (MM test).

Selected active compounds emerging from this MM test are further tested in a secondary test system (Ag test). One of the various tests in this system involves determining whether these compounds will be effective against chloroquine-resistant and multiple drug-resistant parasites **in vivo**.

Other studies involve **1)** determining the best route, vehicle and time to administer a compound to obtain the best suppressive and curative antimalarial activity, **2)** detecting synergistic activity between compounds, **3)** attempts to reverse chloroquine resistance, **4)** induction of resistance to specific compounds in a systematic method, **5)** determining if the administration of pro-oxidant compounds would increase the antimalarial activity of drugs, and **6)** altering the antioxidant status of the host while changing the fatty acid profile of the host red blood cell or parasite membranes rendering them more labile to lipid peroxidation with the ultimate destruction of the parasite.

Chemotherapeutic control of African sleeping sickness caused by **Trypanosoma rhodesiense** or **Trypanosoma gambiense** in humans has not been achieved. This leaves over 20 million people exposed to this organism resulting in serious morbidity and increasing mortality rates. Currently available drugs are ineffective due to a combination of drug-resistant trypomastigotes and severe drug toxicity problems. New compounds are needed to combat this disease so 360 compounds were tested in a primary test (RR test) and 91 compounds against 3 drug-resistant lines.

SCREENING PROCEDURE FOR ASSESSING THE BLOOD SCHIZONTICIDAL ANTIMALARIAL ACTIVITY OF CANDIDATE COMPOUNDS IN PLASMODIUM BERGHEI INFECTED MICE

This mouse malaria (MM) test system was designed to identify new compounds active against asexual blood stages of malaria. Using mice from our breeding colony and a standard inoculum of **P. berghei** it has been possible to produce a consistent disease fatal to 100% of the untreated animals within 6 to 7 days. Active compounds extend the survival time or cure infected mice.

An established disease is less responsive to treatment than a disease in the early stages of development, therefore treatment was deliberately withheld until a moderately high degree of parasitemia was evident. Test compounds were administered subcutaneously (SC) in a single dose on the third day postinfection, at which time a 10-15% parasitemia had developed. A similar procedure was followed for the oral (PO) administration of selected active compounds.

A compound was classified as "active" if it suppressed the disease and produced and unquestionably significant increase, 100% or more, in the life span of the treated animals over that of the untreated infected controls. A compound was considered to be "curative" if the treated animals remained alive for 60 days after infection with **P. berghei**. Compounds not meeting one of the above requirements were considered "inactive".

The severity of the challenge set up in the MM test system enhances the reliability of our evaluation and the antimalarial potential of the compounds selected for intensive preclinical studies.

METHODS

ANIMAL HOSTS

The total supply of animals needed to screen candidate compounds was obtained from our breeding colony of CD-1 Swiss mice (**Mus musculus**). Test animals weighed 18-20 grams. Weight variations in any given experimental or control group were carefully limited to within 2 to 3 grams. In any given test all animals were approximately the same age.

Animals on test were housed in metal-topped plastic cages, fed a standard laboratory diet and given water **ad libitum**. Once the infected mice had been administered the drug, they were placed in a room maintained at 28.8°C(±2°C), with a relative humidity of approximately 66%.

TEST PROCEDURE

Test animals received an intraperitoneal (IP) injection of approximately $6X10^5$ parasitized erythrocytes drawn from donor mice infected 4 days earlier with **P. berghei**. The donor strain was maintained by passage every 4 days in separate groups of mice inoculated with 0.2 cc of a 1:435 dilution of heparinized heart blood.

To check factors such as changes in the infectivity of our **P. berghei** strains or in the susceptibility of the host, 1 group of mice, which served as the negative control, was infected but not treated. To determine the effect that a drug exerted upon a malarial infection, 2 parameters were measured; the first was an increase in survival time, the second concerned curative action. For comparative purposes, 1 standard compound, pyrimethamine, was administered at 1 level (120 mg/kg) to a group of 15 mice. Pyrimethamine served as a positive control, producing a definite increase in survival time and curative effects. Another function of the positive control involved monitoring 3 procedures; the drug weighing, the preparation of drug solutions and suspension, and the administration of drugs.

DRUG ADMINISTRATION

Test compounds were dissolved or suspended in peanut oil before they were administered SC. Compounds to be administered PO were mixed in an aqueous solution of 0.5% hydroxyethylcellulose-0.1% Tween-80 (HEC).

Treatment consisted of a single dose given SC or PO 3 days postinfection. At the time of treatment a 10-15% parasitemia had developed. Although the disease was well established, it had not yet caused sufficient debility to affect an evaluation of the test compound's toxicity.

Deaths that occurred before the sixth day, when untreated infected controls began to die, were regarded as the result of a compound's toxic effect and not as the result of action by the infecting parasite.

Each compound was initially administered in 3 graded doses, diluted 4-fold, to groups of 5 mice per dose level. The top dose was 640, 320, or 160 mg/kg of body weight depending upon the amount of compound available for testing. Active compounds were subsequently tested at 6 or 9 dose levels, diluted 2-fold from the highest dose. Successive 6-level tests were performed at respectively lower doses until the lower limit of activity was reached, thus establishing a complete dose-response picture for that compound in a rodent system.

A drug that was toxic for the host at each of the 3 levels initially tested was retested at 6 dose levels diluted 2-fold from the lowest toxic dose.

DRUG ACTIVITY

Acceptance of a drug being sufficiently active for detailed studies was predicated on the margin between the maximum tolerated dose (MTD) and the minimum effective dose (MED) producing a significant effect. An MTD is defined as the highest dose up to 640 mg/kg causing no more than 1 of 5 animals to die from drug toxicity. The MED is defined as the minimum dose increasing the life span of treated animals by 100% over the life span of untreated controls.

Clearly inactive compounds were rejected after one test, borderline compounds were characterized by a dose-response curve which established the spread the MTD and the lower limit of activity by determination of drug activity in the dose-level dilution test.

RESULTS

During this year 1,502 compounds were tested for activity against asexual blood stages of ***P. berghei***. There were 167 of these compounds which exhibited antimalarial activity.

A total of 298,590 3-dose level tests were performed from December 1, 1961 through January 31, 1989 **(Table I)**. The number of active compounds during part of this time period are summarized in **Table II.**

The specific test data of all compounds tested, (in malaria and trypanosome-drug screens) many of which are commercially or proprietorial discreet, is transmitted to the WRAIR Drug Development Program Chemical/Biological database.

TABLE I

PLASMODIUM BERGHEI MALARIA IN MICE

Compounds Tested

December 1, 1961 - January 31, 1989

TIME PERIOD	NUMBER OF COMPOUNDS TESTED
February 1, 1988 - January 31, 1989	1,502
February 1, 1987 - January 31, 1988	1,500
February 1, 1986 - January 31, 1987	1,507
February 1, 1985 - January 31, 1986	1,500
October 1, 1983 - January 31, 1985	3,390
October 1, 1982 - September 30, 1983	3,026
October 1, 1981 - September 30, 1982	3,020
October 1, 1980 - September 30, 1981	2,998
October 1, 1979 - September 30, 1980	4,826
October 1, 1978 - September 30, 1979	6,175
October 1, 1977 - September 30, 1978	5,375
June 1976 - September, 1977	7,114
June, 1975 - May, 1976	9,916
June, 1974 - May, 1975	10,604
June, 1973 - May, 1974	11,035
June, 1972 - May, 1973	14,276
June, 1971 - May, 1972	14,874
June, 1970 - May, 1971	18,108
June, 1969 - May, 1970	22,376
June, 1968 - May, 1969	38,150
June, 1967 - May, 1968	40,465
June, 1966 - May, 1967	34,093
June, 1965 - May, 1966	22,731
June, 1964 - May, 1965	13,114
December, 1961 - May, 1964	6,915
TOTAL	**298,590**

TABLE II

PLASMODIUM BERGHEI MALARIA IN MICE

Summary of Active Compounds

June 1, 1970, January 31, 1989

TIME PERIOD	NUMBER OF COMPOUNDS TESTED	NUMBER OF ACTIVE COMPOUNDS
February 1, 1988 - January 31, 1989	1,502	167
February 1, 1987 - January 31, 1988	1,500	327
February 1, 1986 - January 31, 1987	1,507	158
February 1, 1985 - January 31, 1986	1,500	74
October 1, 1983 - January 31, 1985	3,390	205
October 1, 1982 - September 30, 1983	3,026	335
October 1, 1981 - September 30, 1982	3,020	574
October 1, 1980 - September 30, 1981	2,998	359
October 1, 1979 - September 30, 1980	4,826	581
October 1, 1978 - September 30, 1979	6,175	969
October 1, 1977 - September 30, 1978	5,375	1,261
June 1, 1976 - September 30, 1977	7,114	1,124
June 1, 1975 - May 31, 1976	9,916	351
June 1, 1974 - May 31, 1975	10,604	616
June 1, 1973 - May 31, 1974	11,035	394
June 1, 1972 - May 31, 1973	14,276	771
June 1, 1971 - May 31, 1972	14,874	593
June 1, 1970 - May 31, 1971	18,108	805
TOTAL	**120,746**	**7,664**

SECONDARY ANTIMALARIAL SCREENING SYSTEM

INTRODUCTION:

DRUG RESISTANCE

Many **P. falciparum** parasites in various geographic areas of the world do not respond to certain standard antimalarial agents while some of these parasites do not respond to any antimalarial agent (multiple drug-resistance). The different categories of drug resistance found in **P. falciparum** are summarized below;

1) Resistance to 4-aminoquinolines
chloroquine

2) Resistance to arylaminoalcohols
mefloquine (a quinolinemethanol)
halofantrine (a phenanthrenemethanol)

3) Resistance to cinchona alkaloids
quinine

4) Resistance to antifol drugs
pyrimethamine
proguanil
Fansidar®
Fansimef®

5) Resistance to acridines
atebrine

6) Multiple-drug resistance (resistance to two or more of the above compounds).

DRUG ACTIVITY

Toxic reactions in humans can occur with many antimalarials. The following compounds have been shown to cause severe toxic reactions in some patients.

Amodiaquine
Fansidar®
Fansimef®
Mefloquine

Collectively, the several types of resistance impair the effectiveness of all the major available antimalarials. Hence, a tremendous need exists for alternate drugs active against the various type of drug-resistant parasites.

Another approach to antimalarial chemotherapy is by using combinations of synergistically active compounds such as Fansidar® (pyrimethamine plus sulfadoxine), or the triple combination of mefloquine, pyrimethamine, and sulfadoxine (Fansimef®).

Unfortunately both of these combinatorial drug regimens share toxicity problems due to the sulfadoxine component. New synergistically active drug combinations are needed.

The ***in vivo*** discovery that desiparmine could reverse resistance to chloroquine in ***P. falciparum*** prompted us to verify this is a mouse model using ***Plasmodium yoelii***.

The antioxidant status of the hosts blood system is an important component often influencing the degree of parasitemia and the eventual pathology caused by malarial organisms. These natural antioxidants may also antagonize the antimalarial activity of drugs which act via free radical formation as their primary mechanism in killing malarial parasites. Such drugs acting through the generation of free radicals include peroxides, primaquine, and qinghaosu type compounds.

By manipulating the hosts major antioxidative components in the blood (vitamin C and E) in concert with metabolically shifting the fatty acid profile of red blood cell and parasite membranes to an increased level of polyunsaturated (Pufa) omega-3 (n-3) fatty acids, one could render the infected red blood cells and the parasite more susceptible to killing by free radical acting drugs. Several studies attempting to study this three way attack on the parasite (lower antioxidant status while increasing Pufa n-3 levels in red blood cells then treating with free radical acting drug) were undertaken with very successful results.

METHODS

PARASITES

Drug-sensitive lines

Plasmodium berghei KBG-173 (P-line)
Plasmodium yoelii 17 X (X-line)

Drug-resistant lines

P. berghei KBG-173

mefloquine resistant	A-line
chloroquine resistant	C-line
pyrimethamine resistant	M-line
quinine resistant	Q-line
dapsone resistant	S-line
cycloguanil resistant	T-line

P. yoelii 17X

qinghaosu resistant	U-line

ANIMAL HOST

The testing was done in both female and male CD-1 Swiss mice (**Mus musculus**) obtained from our own breeding colony. Four week-old mice were used for most experiments except 3 week-old weanling male mice were used to start the antioxidant studies.

TEST DESIGNS

SINGLE OR MULTIPLE DOSE MODIFIED MM TESTS

A series of artemisinin analogs were administered either once on D+3 or multiple times on days 3, 4 and 5 after inoculation with a regular MM parasite inoculum of $6X10^5$ erythrocytes parasitized with **P. berghei**. In one test mefloquine was administered at low levels at the same time as Na artelinate to see if it would enhance its activity. Blood films were taken weekly starting on the sixth day after infection and continued for a 60 day period. Mice surviving the 60 day challenge were considered cured. Several standard antimalarial compounds were suspended in fat or water soluble solutions and administered once on D+3. In one test mice were treated once on D+3 and after 60 days half the mice were rechallenged while the other half were bled and their blood subinoculated into recipient mice to detect any latent parasites.

6-DAY SUPPRESSIVE TEST

In this basic 6-day suppressive test, mice were divided into groups of 7 and inoculated with $5X10^4$ parasites intraperitoneally (i.p.). For several drug-resistant lines (A, C, Q, S, and U lines) $15X10^6$ parasitized red blood cells are administered i.p. Drugs were

administered twice a day, in a volume of 10 ml/kg on the third, fourth and fifth days after inoculation of parasites. All drugs were mixed in HEC. One group of infected mice received the vehicle alone and served as a negative control group. Blood films were made on the sixth day after inoculation of parasites. Microscopic examination of Giemsa-stained blood smears was made to determine the percentage of cells parasitized. The percent suppression of parasitemia, and significance values for the suppression of parasitemias were then determined. Significance values for the percent suppression of parasitemia were determined by comparing the parasitemia of each treated mouse with the mean parasitemia of the negative control animals. Drug tolerance was reflected by the percent weight change and the proportion of mice that survived treatment.

SYNERGISTIC TEST

Mice were infected i.p. with $5X10^4$ parasitized erythrocytes of the X-line. The drugs were mixed separately then administered either alone or as a mixture orally twice a day on days 3, 4, and 5 after the mice had been infected. The effects were determined from blood smears made 1 day after completion of treatment. The dose suppressing 90% of the parasites (SD_{90}) for 1 drug alone and of the mixture were estimated by plotting parasitemia suppressions on probit-log scale graphs. The analyses for synergism were based upon partitioning of the SD_{90} value of each combination in terms of its components. These components were then compared with the respective SD_{90} values of the corresponding drug alone. If the joint effects were simply additive, each component of a mixture SD_{90} would be expected to be 0.5. If all values were lower than 0.5 the data would indicated synergism. Conversely, if all values were greater than 0.5 the data would indicated antagonism.

REVERSING CHLOROQUINE RESISTANCE

An attempt to reverse chloroquine resistance in a moderately chloroquine-resistant line was done with a combination of desiparmine and chloroquine. The results were determined by comparing the SD_{90} values of the drug combinations with the SD_{90} values of each component administered singly.

OXYGEN RADICALS

A series of compounds which generate oxygen radicals were administered alone or combined with antimalarial agents in order to increase the oxidative stress in the infected red blood cell leading to its eventual lysis and the demise of the parasite.

Selected pro-oxidants were also tested for antimalarial activity. All the compounds were accessed for suppressive activity in the 6-day suppressive test and curative activity was determined by monitoring survival times for 60 days postinfection.

ANTIOXIDANT STUDIES INVOLVING CHANGING FATTY ACID PROFILES IN MEMBRANES

A series of 15 experiments were performed to study the influence of altering the fatty acid profiles of host red blood cells and parasites membranes in infected mice fed vitamin E-deficient diets. Various Pufa high in n-3 fatty acids were used as the dietary fat source in these diets. Suppressive and curative antimalarial activity were determined by monitoring parasitemia levels and mortality data. Several experiments also were designed to study the influence of paraaminobenzoic acid (PABA) and other antioxidants on the growth of malarial parasites.

INDUCTION OF DRUG RESISTANCE

FLOXACRINE ANALOG RACEMATE AND ITS R- AND S-STEREOISOMERS

A comparative study was started in experiment 557 to determine how fast resistance could be attained to each of the following compounds;

1. BL21100 A floxacrine analog racemate,
2. BL34170 The R-Stereoisomer of BL21100,
3. BL29759 The S-Stereoisomer of BL21100.

The drug-sensitive line of **P. yoelii** was used to start the induction of resistance. Each of three compounds was first administered at the same 7 dose levels (2, 1, 0.85, 0.5, 0.38, 0.25, 0.125 mg/kg/day) for 3 consecutive days bid commencing on D+3 after infection with $5X10^4$ parasitized erythrocytes. Blood films were made on D+7 and the mouse at the highest dosage level with a parasitemia of 1-5% was used as a donor mouse. This procedure was repeated weekly using the dose level passed to be the second lowest dose (X) and then the drug was increased for the next pass according the following increments.

8X
4X
3X
2X
1.5X
X
0.5X
0 - No drug.

By using this schedule a standardized procedure was used to assess the speed that resistance to each compound was attained.

QINGHAOSU

Starting with a drug-sensitive line of **P. yoelii** qinghaosu was administered at increased levels each week until resistance to 256 mg/kg/day was achieved.

RESULTS

SINGLE OR MULTIPLE DOSE MM TESTS

In test 7 arteether suspended in sesame oil and administered once was more active SC than PO (**Table III**). Curative activity was obtained down to the 15 mg/kg SC while 480 mg/kg were needed to cure mice orally. In test 8 two of three artemisinin analogs suspended in HEC and administered orally on D+3, 4 and 5 exhibited curative activity. BL55811 cured mice at 80 mg/kg. BL55802 was not curative at a top dose of 320 mg/kg (**Table IV**). In test 11 Na artelinate administrated once in HEC PO on D+3 exhibited suppressive activity at 80 mg/kg with no cures at a top dose of 160 mg/kg (**Table V**). In experiment 12 when mefloquine was administered at the same time as Na artelinate an increase in suppressive and curative activity was observed at certain drug levels (**Table VI**).

Two trioxanes mixed with tyloxapol (a surfactant) then suspended in 2% methyl cellulose and administered once SC and PO on D+3 were active. BL52276 was suppressive at 160 mg/kg SC and 640 mg/kg PO while BL52285 was suppressive at 640 mg/kg SC but not PO at this level (**Table VII**).

Chloroquine and primaquine suspended in 5 different vehicles and administered either SC or PO exhibited different toxicities and activities. Chloroquine was less toxic SC in SuperMax EPA than in peanut oil or menhaden oil and was not toxic PO at 640 mg/kg in a water soluble vitamin E solution (Liqui-E) but toxic at 640 mg/kg in HEC (**Table VIII**). Primaquine was also less toxic SC in SuperMax EPA and PO than in Liqui-E. The suppressive activity of both chloroquine and primaquine were similar SC and PO in the different vehicles (**Table VIII**).

Four compounds administered SC in peanut oil or HEC and PO in HEC exhibited different toxicity and activity. Dapsone was more toxic SC in peanut oil than in HEC. AV05287 and dapsone were more active SC in peanut oil than HEC while AV40731 and BE85702 were similar in activity (**Table IX**). Mice treated with 1 of 3 compounds once on D+3 were divided into 2 groups on D+60. One group were bled and their blood subinoculated into normal recipient mice to see whether parasites were still present. The other group of mice were reinoculated with parasites. Mice receiving WR158122 were refractory to rechallenge and exhibited no parasites in the subinoculated mice (**Table X**). Half the mice receiving mefloquine were positive upon rechallenge but no parasites were found in the subinoculated mice. Mice receiving quinacrine exhibited parasites when rechallenged but only one level (160 mg/kg) still retained parasites at D+60 when the blood was subinoculated.

6-DAY SUPPRESSIVE TESTS

In experiment 530 one sustained release formulation of artemisinin (QHS) BL47408 was more active then another BL47417 (**Table III**). The dapsone-resistant line was checked for cross resistance to quinine, primaquine, pyrimethamine and sulfadiazine. No cross resistance was observed in this line resistant to 256 mg/kg/day of dapsone. A vial of the mefloquine-resistant line stored in liquid nitrogen for several years was removed and red blood cells were inoculated into mice. One week after being in mice the parasites susceptibility to several antimalarial was determined. It was found to have lost some of its resistance to mefloquine and its cross resistance to chloroquine, quinine and quinacrine was less. It was still as susceptible to sulfadoxine, pyrimethamine, dapsone, 4-methyl primaquine, and WR158122 as a drug-sensitive line.

In experiment 562 chloroquine and mefloquine were administered against 3 different resistant lines, Flox 1 (resistant to a floxacrine analog WR243251), Flox 2 (resistant to the R-stereoisomer of WR243251 which is WR250547) and to Flox 3 (resistant to the S-stereoisomer of WR243251 which is WR 250548). Chloroquine was cross resistant to each of these 3 lines while mefloquine remained active against Flox 1 and 2 but less active against Flox 3 (the S-stereoisomer).

SYNERGISTIC TESTS

STEREOISOMERS OF A FLOXACRINE ANALOG

A floxacrine analog (WR243251) has been resolved into its R-(WR250547) and S-(WR250548) stereoisomers. These two stereoisomers were found to interact synergistically in suppressing and curing malarial infections of **P. yoelii** in experiments 546 and 547.

Na ARTELINATE AND MEFLOQUINE

Synergistic curative activity was observed between these two compounds against **P. yoelii**.

REVERSING CHLOROQUINE RESISTANCE

Desiparmine was administered 3 times a day on D+3, 4 and 5 while chloroquine was administered twice a day on D+3, 4, and 5 to mice infected with **P. yoelii** which contains a small population of highly chloroquine-resistant parasites. No reversal of chloroquine resistance was observed.

OXYGEN RADICALS

The administration of menhaden oil just prior to giving 4-methyl primaquine or QHS did not alter their antimalarial activity (**Table XIV**). The activity of primaquine was not altered by the coadministration of TBHQ, $CuSo_4$ or $FeSO_4$. When a water soluble vitamin E solution was used as a vehicle for suspending primaquine, chloroquine or QHS, their antimalarial activity was similar to that obtained when suspended in HEC. Various plant oils (peanut, sunflower, olive, corn, canola, safflower) administered to infected mice did not alter the course of infection. A free radical generator reduced the antimalarial activity of alloxan. Primaquine and 4-methyl primaquine did not have their antimalarial activity altered alloxan. Vitamin E added to primaquine, chloroquine, and artelenic acid did not alter their antimalarial activity.

ANTIOXIDANT STUDIES INVOLVING CHANGING FATTY ACID PROFILES IN MEMBRANES

EXPERIMENT 10. Effect of DPPD and TBHQ on malaria in vit E deficient mice fed menhaden oil.

Diphenylparaphenyldiamine (DPPD) and tert-butylhydroquinone (TBHQ) are two antioxidants added to the mouse diets which prevented the diets from becoming rancid but did not interfere with the development of suppression of malaria in the vitamin E-deficient diets.

EXPERIMENT 12. Effect of two plant oils (rapeseed and linseed) on the development of malaria.

Rapeseed oil (canola) contains 10% n-3 fatty acids while linseed oil has a 53% level. Only linseed oil without vitamin E cured the mice.

EXPERIMENT 14. Chloroquine-resistant parasites suppressed in mice fed vitamin E-deficient menhaden oil diet.

Both drug-sensitive and chloroquine-resistant parasites can be suppressed and eliminated by a vitamin E-deficient menhaden oil diet.

EXPERIMENT 15. Chloroquine-resistant parasites are eliminated in vitamin E-deficient mice fed a diet containing 4% menhaden oil and 1% corn oil.

The addition of 1% corn oil, as a source of essential fatty acids (n-6 group), to a 4% menhaden oil diet did not interfere with the curative activity obtained with vitamin E-deficient diet.

EXPERIMENT 17. Effect selenium depletion on chloroquine-resistant parasite growth.

Depletion of selenium did not interfere with the growth of chloroquine-resistant malaria while a depletion of vitamin E did.

EXPERIMENT 18. Influence of PABA on the growth of malaria in mice fed a vitamin E supplemented diet containing menhaden oil.

Twenty mg/kg of paraaminobenzoic (PABA) added to a vitamin E supplemented menhaden oil diet allowed 50% of the parasites to grow.

EXPERIMENT 20. Drug-sensitive parasites are eliminated in vitamin E deficient mice fed a diet containing 4% linseed oil and 1% corn oil.

The 1% corn oil (containing no n-3 fatty acids) added to the 4% linseed oil (containing 53% n-3 fatty acids) used as a fat source in a vitamin E-deficient diet did not alter its antimalarial activity.

EXPERIMENT 21. The effect of 1X, 10X, 50X TBHQ on the antimalarial activity of a vitamin E-deficient menhaden oil diet.

Too much TBHQ (50X) interfered with the antimalarial effect of the diets while those containing 1X or 10X did not alter the antimalarial activity.

EXPERIMENT 22. The antimalarial activity of anchovy oil in a vitamin E-deficient diet.

Anchovy oil containing similar levels of n-3 fatty acids as menhaden oil suppressed the malaria growth in a vitamin E-deficient diet comparable to that obtained with menhaden oil.

EXPERIMENT 23. The antimalarial activity of borage oil and a ethyl ester concentrate of fish oil.

Borage oil contains gamma linolenic fatty acid (an n-6 form) which suppressed the parasitemia for one week but produced no cures when used as the fat source in a vitamin E-deficient diet. A ethyl ester concentrate of fish oil containing 68% total eicosapentaenoic acid (EPA) plus docosahexaenoic acid (DHA) did not exert an increase in antimalarial activity when compared with regular menhaden oil which contains 20% EPA + DHA.

EXPERIMENT 24. The effect of PABA on the growth of malaria in mice fed a menhaden oil diet deficient or supplemented with vitamin E.

A 20 mg/kg level of PABA included in a menhaden oil diet was enough to allow the malaria to grow and not be a limiting factor in the course of this experiment.

EXPERIMENT 25. The influence of a menhaden oil diet deficient in vitamin E and PABA on malarial parasites when started on -7, 0 or +3 days postinfection.

A menhaden oil diet deficient in both vitamin E and PABA cured mice even when started 3 days postinfection with **P. yoelii**.

EXPERIMENT 26. The effect of salmon oil and flaxseed oil on **P. yoelii** infections in vitamin E-deficient mice.

Salmon oil in a vitamin E-deficient diet cured mice to the same degree as menhaden oil. Flaxseed oil cured about half the mice in a vitamin E-deficient diet.

EXPERIMENT 27. The influence of a linseed oil diet deficient in vitamin E and PABA on malarial parasites when started on -7, 0, or +3 days postinfection.

A linseed oil diet deficient in both vitamin E and PABA cured mice even when started 3 days postinfection with **P. yoelii**.

EXPERIMENT 28. The effect of a menhaden oil diet deficient in vitamin E on Qinghaosu-resistant parasites.

Mice infected with Qinghaosu-resistant parasites were cured when fed a menhaden oil diet deficient in vitamin E.

INDUCTION OF DRUG RESISTANCE

FLOXACRINE ANALOG RACEMATE AND ITS R- AND S-STEREOISOMERS

Resistance increased weekly with each compound through the fourth pass with resistance to the R-stereoisomer developing at a slower rate for the first 3 passages than the racemate or S-stereoisomer. However, by the fourth pass resistance was high for

each compound. Parasites were present at 96 mg/kg level for the racemate, 64 mg/kg for the R-stereoisomer and 128 mg/kg for the S-stereoisomer. One week later when the fifth pass was to take place there were no parasites in any of the mice under drug pressure at levels of 128, 96, or 64 mg/kg. Therefore parasites from infected non-treated control mice were passed to restart the three resistant lines. Resistance reappeared by the sixth pass with the S-stereoisomer, however, resistance was slower to obtain with the racemate and R-stereoisomer. Resistance to the racemate increased gradually until the seventheenth pass then parasites resistant to 64 mg/kg were obtained. The R-stereoisomer was more difficult to reinduce resistance and it wasn't until the twenty-fourth pass when resistance was noted once again.

Resistance to the R-stereoisomer of the floxacrine analogue racemate developed slower than the S-stereoisomer or the parent racemate compound. When resistance waned at the fifth passage it was more difficult to reinduce resistance to the R-stereoisomer than the S-stereoisomer or racemate.

QUINGHAOSU

Resistance increased weekly to QHS until a top dose of 256 mg/kg/day would be tolerated. This took approximately 6 weeks.

TABLE III

CURATIVE ACTIVITY OF ARTEETHER MIXED IN SESAME OIL AND ADMINISTERED ONCE EITHER SUBCUTANEOUSLY OR ORALLY

TEST NO.	COMPOUND NAME OF BOTTLE NO.	MG/KG ONCE ON D+3	VEHICLE	ROUTE	NO. MICE ALIVE D+60/TOTAL
7	Arteether BL 51082	480		SC	4/5
		240			5/5
		120	Sesame		5/5
		60	oil		4/5
		30	(BL 51091)		3/5
		15			1/5
		7.5			0/5
	Arteether BL51082	960		PO	3/5
		480	Sesame		4/5
		240	oil		0/5
		120	(BL 51091)		0/5
		60			0/5
		30			0/5
		15			0/5

TABLE IV

SUPPRESSIVE AND CURATIVE ACTIVITY OF 3 ARTEMISININ ANALOGS MIXED IN HEC AND ADMINISTERED SUBCUTANEOUSLY

TEST NO.	BOTTLE NO.	MG/KG DAY ONCE A DAY D+3,4,5	MST* (DAYS)	NO. MICE ALIVE ON D+60
8	BL 55811	320	7	4/5
		80	>60	5/5
		20	17	1/5
	BL 55802	320	7	0/5
		80	6	0/5
		20	6	0/5
	BL 55795	320	>60	5/5
		80	20	0/5
		20	6	0/5

* MST = Mean Survival time

TABLE V

SUPPRESSIVE AND CURATIVE ACTIVITY OF Na ARTELINATE MIXED IN HEC AND ADMINISTERED ORALLY

TEST NO.	COMPOUND NAME OR BOTLLE NO.	MG/KG/DAY ON D+3,4,5	MST* (DAYS)	NO. MICE ALIVE D+60 TOTAL
11	Na Artelinate BL 55866	160	15	0/5
		80	14	0/5
		40	10	0/5
		20	10	0/5
		10	8	0/5
		5	6	0/5
		2.5	6	0/5

* MST = Mean Survival time

TABLE VI

SUPPRESSIVE AND CURATIVE ACTIVITY OF Na ARTELINATE AND MEFLOQUINE MIXED IN HEC AND ADMINISTERED ORALLY EITHER SINGLY OR IN COMBINATION

TEST NO.	COMPOUND	MG/KG/DAY ON D+3,4,5	MST* (DAYS)	NO. MICE ALIVE D+60/TOTAL
12	Na Artelinate	160	15	0/5
		80	15	0/4
		40	15	0/5
		20	8	0/5
		10	7	0/6
		5	7	0/5
	Mefloquine	160	23	2/5
		80	18	2/5
		40	23	0/5
		20	18	1/5
		10	16	1/5
		5	14	0/5
	Na Artelinate + Mefloquine			
		40 + 10	25	1/5
		20 + 10	24	1/5
		10 + 10	17	1/5
		5 + 10	18	0/5
		40 + 5	18	1/5
		20 + 5	18	2/5
		10 + 5	17	0/5

* MST = Mean Survival time

TABLE VII

SUPPRESSIVE AND CURATIVE ACTIVITY OF 2 TRIOXANES

TRIOXANES	MG/KG/DAY ONCE ON D+3,4,5	SC MST* (DAYS)	SC NO. MICE ALIVE D+60	PO MST* (DAYS)	PO NO. MICE ALIVE D+60
BL 52276	640	17	2	15	0
	320	15	0	10	0
	160	13	0	8	0
	80	8	0	7	0
	40	6	0	6	0
	20	6	0	6	0
	10	6	0	6	0
BL 52285	640	12	0	7	0
	320	9	0	6	0
	160	7	0	6	0
	80	6	0	6	0
	40	6	0	6	0
	20	6	0	6	0
	10	6	0	6	0

The wetting agent tyloxapol (a surfactant) was added to each compound before it was suspended in 2% methyl cellulose and administered once on D+3.

* MST = Mean Survival time

TABLE VIII

COMPARATIVE SUPPRESSIVE ACTIVITY OF CHLOROQUINE AND PRIMAQUINE MIXED IN VARIOUS VEHICLES

COMPOUND	MG/KG ONCE ON D+3	MEAN SURVIVAL TIME (DAYS) SC PEANUT OIL	SC SUPER MAX EPA	SC MENHADEN OIL	PO LIQUID VIT E	PO HEC TWEEN
Chloroquine	640	3^{5T}	3^{5T}	3^{5T}	18	17^{4T}
	320	3^{5T}	23^{2T}	3^{5T}	14	15
	160	18^{4T}	14	18^{3T}	15	13
	80	13	11	11	12	14
	40	12	12	13	12	10
	20	9	8	9	9	10
	10	7	8	7	9	9
	5	7	6	7	8	7
	2.5	6	6	6	6	7
Primaquine	640	9^{4T}	14^{1T}	18^{2T}	3^{5T}	3^{5T}
	320	8^{3T}	18	14	8^{4T}	21^{4T}
	160	12^{1T}	12	21	16	10^{1T}
	80	10	11	11	14	10
	40	9	10	11	12	10
	20	7	8	8	8	8
	10	6	7	6	8	8
	5	7	6	6	7	7
	2.5	6	6	6	6	7

TABLE IX

SUPPRESSIVE AND CURATIVE ANTIMALARIAL ACTIVITY OF 4 COMPOUNDS

		SC				PO	
		PEANUT OIL		HEC		HEC	
BOTTLE NO.	MG/KG ONCE ON D+3	MST	MICE ALIVE D+60	MST	MICE ALIVE D+60	MST	MICE ALIVE D+60
AV 05287	640	22	3	15	3	10	0
	320	16	3	15	1	7	0
	160	13	1	11	0	7	0
	80	14	0	9	0	8	0
	40	10	0	8	0	7	0
	20	8	0	7	0	7	0
AV 40731	640	18	3	20	2	11	0
	320	18	0	17	0	8	0
	160	11	0	11	0	10	0
	80	8	0	10	0	8	0
	40	7	0	6	0	7	0
	20	6	0	6	0	7	0
BE 85702	40	29	2	36	3	32	2
	20	21	1	27	3	23	1
	10	16	0	26	0	21	1
	5	13	0	15	0	17	0
	2.5	6	0	7	0	16	0
	1.25	6	0	6	0	6	0

TABLE IX (Cont.)

SUPPRESSIVE AND CURATIVE ANTIMALARIAL ACTIVITY OF 4 COMPOUNDS

		SC				PO	
		PEANUT OIL		HEC		HEC	
BOTTLE NO.	MG/KG ONCE ON D+3	MST	MICE ALIVE D+60	MST	MICE ALIVE D+60	MST	MICE ALIVE D+60
Dapsone	640	19	3[1T]	>60	5	18[2T]	0
ZB 69096	320	26	2[1T]	>60	5	16	2
	160	24	3	23	2	15	0
	80	17	1	17	2	14	0
	40	15	0	16	0	12	0
	20	16	0	13	0	10	0
	10	12	0	13	0	8	0
	5	12	0	9	0	7	0
	2.5	9	0	6	0	7	0

T = Toxic dose

TABLE X

SUBINOCULATION AND RECHALLENGED DATA FOR MICE SURVIVING 60 DAYS POSTINFECTION

COMPOUND BOTTLE NO.	MG/KG ONCE ON D+3	ROUTE AND VEHICLE	MST (DAYS)	NO.MICE ALIVE D+60 TOTAL	RECHALLENGED MICE POSITIVE	SUBINOCULATED MICE POSITIVE RECIPIENT PARASITES
158122	320	SC	>60	5/5*	0/5	
AY 65859	160	HEC	>60	5/5*	0/5	
		Tween				
	80		>60	5/5*	0/5	
	320		>60	5/5@		0/5
	160		>60	5/5@		0/5
	80		>60	5/5*		0/5
Mefloquine	640	SC	24	4/5*	2/4	
BH 10371	320	Peanut	24	2/5*		
	160	Oil	26	0/5*		
	640		30	0/5@		
	320		24	1/5@		0/1
	160		19	1/5@		0/1
Chloroquine	320	PO	18	0/5		
AU 29291	160	HEC	12	0/5		
	80	Tween	13	0/5		
	320		16	0/5		
	160		15	0/5		
	80		14	0/5		
Quinacrine	640	PO	>60	5/5*	5/5	
AU 96336	320	HEC	>60	5/5*	4/5	
	160		24	4/5*	2/2	
	640		>60	5/5@		0/5
	320		>60	5/5@		0/5
	160		20	3/5@		2/3

* = These mice were then rechallenged with a regular parasite inoculum on D+60.

@ = 0.25 cc whole blood was removed from these mice and subinoculated into normal mice.

TABLE XI

SUPPRESSIVE ANTIMALARIAL ACTIVITY OF 2 SUSTAINED RELEASE FORMULATIONS OF QINGHAOSU (QHS), COMPARED TO QHS, AND ARTEETHER VS P. YOELII

TEST NO.	COMPOUND OR BOTTLE NO.	MG/KG/DAY	% SUPPRESSION D+6
530	BL 47408	160	99
	Sustained release	40	86
	QHS	10	75
		2.5	0
	BL 47417	160	95
	Sustained release	40	86
	QHS	10	0
		2.5	14
	QHS	160	99
		40	89
		10	4
		2.5	0
	Arteether	160	100
		40	100
		10	96
		2.5	14

TABLE XII

ACTIVITY OF ANTIOXIDANTS, PRO-OXIDANTS AND OILS ALONE ON MALARIA OR COMBINED WITH DRUGS

EXPERIMENT	COMPOUNDS	PARASITE LINE	ANTIMALARIAL ACTIVITY		
			INCREASED	DECREASED	NO CHANGE
529	4 CH_3 Primaquine + Men Oil	X			+
	QHS + Men Oil	X			+
531	Primaquine + TBHQ	X			+
	Primaquine + $CuSO_4$	X			+
	Primaquine + $FeSO_4$	X			+
532	Primaquine in Liquid Vit E	X			+
	Primaquine in HEC	X			+
	Chloroquine in Liquid Vit E	X			+
	Chloroquine in HEC	X			+
	QHS in Liquid Vit E	X			+
	QHS in HEC	X			+
533	Peanut oil	X			+
	Sunflower oil	X			+
	Olive oil	X			+
	Menhaden oil	X			+
	Canola oil	X			+
	Safflower oil	X			+
	Alloxan + Free Radical Generator	X		+	+
535	Primaquine + Alloxan	X			+
	4-CH_3 Primaquine + Alloxan	X			+

TABLE XII (Cont.)

EXPERIMENT	COMPOUNDS	PARASITE LINE	ANTIMALARIAL ACTIVITY		
			INCREASED	DECREASED	NO CHANGE
536	Menhaden oil	C			+
	Cod liver oil	C			+
	Peanut oil	C			+
	ProMega	C			+
	Liquid Vit E	C			+
	Chloroquine + $FeSO_4$	C			+
	Chloroquine + $CuSO_4$	C			+
537	Vitamin E	X			+
	Menhaden oil + $FeSO_4$	X			+
	Menhaden oil + $CuSO_4$	X			+
	Malonaldehyde PO	X			+
	Malonaldehyde IP	X			+
538	Menhaden oil + Alloxan	X			+
	Canola oil + Alloxan	X			+
	TBHQ + Alloxan	X			+
539	Vitamin A PO	X			+
	Vitamin A SC	X	+		
	Vitamin B_{12} PO	X			+
	Vitamin B_{12} SC	X			+
	Vitamin E PO	X			+
	Vitamin E SC	X			+
544	QHS + Vit E® (294 mg)	X			+
	Altelinic acid + Vit E® (294 mg)	X			+
	Primaquine + Vit E® (294 mg)	X			+

TABLE XII (Cont.)

EXPERIMENT	COMPOUNDS	PARASITE LINE	ANTIMALARIAL ACTIVITY		
			INCREASED	DECREASED	NO CHANGE
548	QHS + Vit E (20 mg)*	X			+
	QHS + Vit E (200 mg)*	X			+
	Artelenic acid + Vit E (20 mg)*	X		+	
	Artelenic acid + Vit E (200 mg)*	X			+
558	Ethyl linolenate	X			+
	Linolenic acid	X			+
582	Na artelinate + Vit E (200 mgs)	X			+
	Na artelinate + Vit E (400 mgs)	X			+

@ = Vit E was mixed with the drugs.
* = Vit E given once a day on days 2, 3, 4, and 5.
• = Vit E given once a day on days 3, 4, and 5.
x = Vit E was given once a day in peanut oil on days 0, 1, 2, 3, 4, 5.

A SCREENING PROCEDURE FOR THE EVALUATION OF TRYPANOCIDAL ACTIVITY OF CANDIDATE COMPOUNDS IN TRYPANOSOMA RHODESIENSE INFECTED MICE

INTRODUCTION

The World Health Organization estimates that there were about 20,000 new cases of African trypanosomiasis last year with 45 million peole living in endemic areas. The various species of the vector (**Glossina**) are found over 4.5 million square miles in Africa. African trypanosomiasis has a very high mortality rate and has considerable importance as a public health problem, especially in this age of increasing foreign travel. The few drugs available for use today are toxic and parasite resistance to these agents is commonly found.

No new trypanocidal drugs have been introduced since the synthesis of pentamidine in 1939. Four drugs are currently available for the treatment of human trypanosomiasis caused by **Trypanosoma rhodesiense** or **Trypanosoma gambiense**. Two of these drugs, suramin and pentamidine are used in the treatment of the blood parasite (trypomastigote), but lack of efficacy in the treatment of central nervous system infections with trypomastigotes. Melarsoprol and nitrofurazone are used for the treatment of trypomastigotes in the central nervous system.

All of these drugs have severe side effects resulting in poor therapeutic indices. The use of suramin may lead to nausea, vomiting, shock and loss of consciousness. It can also cause exfoliate dermatitis, albuminuria, hematuria and ultimately renal failure. Pentamidine's use may lead to fatal hypertension, hypoglycemia, diabetes and renal dysfunction. Administration of melarsoprol may lead to lethal encephalopathy and in 10 to 15 percent degeneration of the seminiferous tubules. This drug is also associated with causing hemolytic anemia in glucose 6-phosphate dehydrogenase deficient patients.

Compounding the problem of low therapeutic indices is the problem of trypanosomal drug resistance. Human trypanosome strains are commonly resistant to at least 1 chemotherapeutic agent. With some patients, their infection is resistant to 2 or more antitrypanosomal drugs.

Therefore, there is a definite need to develop and test compounds that are potentially active against resistant strains of **T. rhodesiense** and that are less toxic than the existing drugs. Further testing also needs to be done using different routes of administration and combinations of 2 or more drugs.

The test system described herein was developed specifically to evaluate the trypanosomal activity of large numbers of candidate compounds. Based on blood induced **T. rhodesiense** infections in mice, it acts as a primary screen or as a secondary screen and/or confirmatory test. This test gives a precise quantitative evaluation of chemical compounds that demonstrate potentially useful therapeutic and/or prophylactic activity in **T. rhodesiense** infections. Consequently, it is also a helpful guideline in the synthesis of new related active agents.

All candidate compounds are obtained from the chemical inventory of the Division of Experimental Therapeutics at the Walter Reed Institute of Research.

METHODS

ANIMALS HOSTS

CD-1 Swiss mice (**Mus musculus**) used in this screening procedure weighed 25 to 28 grams with weight variation in any given experimental or control group carefully limited to 3 grams. Male and female mice approximately the same age were used.

Animals were housed in metal-topped plastic cages, fed a standard laboratory diet and given water **ad libitum**. After drug treatment, mice were kept in a room maintained at a temperature of 28.8°C (±2°C) with a relative humidity of 66% (±2%).

INOCULATION OF PARASITES

Test animals received an intraperitoneal injection of 0.2 cc of a $1.5X10^4$ dilution of heparinized heart blood drawn from a donor mouse infected 3 days earlier (approximately $1.3X10^4$-$1.7X10^4$ trypomastigotes).

The donor line was maintained by 3-day blood passes; each animal received 0.1 cc of a $1:1.5X10^4$ dilution of heparinized heart blood drawn from a mouse harboring a 3-day infection.

One group of infected, untreated mice was included as a negative control to check both the infectivity of the **T. rhodesiense** (CT-Wellcome strain) and the susceptibility of the murine host. In order to determine the effect a drug exerted on a trypanosome infection, 2 parameters were measured; **1)** the increase

in mouse survival time and **2)** drug curative action. For comparative purposes, 2 standard antitrypanosomal compounds, stilbamidine isethionate and 2-hydroxystilbamidine isethionate, were administered subcutaneously at one dose (26.5 mg/kg) to separate groups of 10 mice each. The same positive controls were administered orally at 53 mg/kg when compounds were tested orally. These 2 diamidines served as positive controls, producing definite increase in survival time and curative effects.

DRUG ADMINISTRATION

Test compounds were dissolved or suspended in peanut oil before they were administered subcutaneously. Compounds to be administered orally were mixed in an aqueous solution of HEC.

Treatment consisted of a single dose, given subcutaneously or orally, 2 to 3 hours after the injection of parasites. Deaths that occurred before the 4th day, when untreated infected controls began to die, were regarded as a result of toxic action by the drug, not the lethal effects of the parasites.

Each compound was initially administered in 3 graded doses diluted 4-fold to groups of 5 mice per dose level. The top dose was either 424, 212, or 106 mg/kg, depending on the amount of compound available for testing. Active compounds were subsequently tested at 6 dose levels, diluted 2-fold from the highest dose. If necessary, successive 6-level tests were performed at respectively lower doses until the lower limit of activity was reached.

A drug that was toxic for the host at each of the 3 levels initially tested was retested at 6 dose levels diluted 2-fold from the lowest toxic dose.

DRUG ACTIVITY

Acceptance of a drug as being sufficiently active for detailed studies was predicated on the margin between the maximum tolerated dose (MTD) and the minimum effective dose (MED) producing a significant effect. A MTD is defined as the highest dose up to 424 mg/kg causing no more than 1 of 5 animals to die from drug activity. The MED is defined as the minimum dose increasing the life span of treated animals by 100% over the live span of untreated infected controls.

Clearly inactive compounds were rejected after 1 test and border-line compounds after 2 tests. Active compounds were characterized by dose-response curves, which established the spread between the MTD and the lower limit of activity by a determination of drug activity in the dose-level dilution tests. Treated animals alive at the end of 30-days were considered cured.

RESULTS

CONTROL

Mice inoculated with trypomastigotes but receiving no drug (negative control group) all routinely died within 4 to 5 days. Mice serving as positive controls, receiving 26.5 mg/kg of stilbamidine, or 26.5 mg/kg of 2-hydroxystilbamidine, usually survived for the duration of the experiment (30 days).

COMPOUNDS TESTED

Data for all the new compounds is summarized in **Table XIII.** There were 55 active compounds out of 360 tested.

TABLE XIII

SUMMARY OF TRYPANOSOMA RHODESIENSE DRUG-SCREEN TEST

REPORT PERIOD	NUMBER OF THREE-LEVEL TESTS	NUMBER OF COMPOUNDS TESTED	TOTAL NUMBER OF ACTIVE COMPOUNDS	NUMBER OF COMPOUNDS ACTIVE BY ROUTE OF ADMINISTRATION		
				ORAL ONLY	S.C. ONLY	ORAL AND S.C.
2/1/88 - 1/31/89	360	360	55	0	55	0
2/1/87 - 1/31/88	1,004	666	132	0	132	0
2/1/86 - 1/31/87	1,006	794	89	8	31	50
2/1/85 - 1/31/86	1,060	816	67	21	31	15
10/1/83 - 1/31/85	2,372	2,056	142	1	141	0
10/1/82 - 9/30/83	2,069	1,788	82	0	75	7
10/1/81 - 9/30/82	1,994	1,960	67	3	60	2
10/1/80 - 9/30/81	2,043	1,222	62	6	50	6
10/1/79 - 9/30/80	4,780	3,462	88	3	78	7
10/1/78 - 9/30/79	3,158	2,783	125	7	116	2
10/1/77 - 9/30/78	4,025	3,032	77	9	54	14
6/1/76 - 9/30/77	4,235	4,235	396	17	270	109
6/1/75 - 5/31/76	1,653	1,653	257	59	198	
6/1/74 - 5/31/75	1,826	1,826	298	73	225	
6/1/73 - 5/31/74	1,581	1,581	185	93	92	
8/1/72 - 5/31/73	3,030	3,030	68			
TOTAL	**36,136**	**31,264**	**2,190**	**300**	**1,608**	**212**

DRUG-RESISTANT TRYPANOSOME TEST

INTRODUCTION

Drug-resistant parasites of **T. rhodesiense** and **T. gambiense** in humans severely complicate the chemotherapy approach to this disease. Pentamidine, the best available drug to treat the blood stream trypomastigotes stages of both species of African trypanosomes, is no longer effective in many areas of Africa because resistant parasites are commonly found. Suramin is the other drug used against the trypomastigotes of **T. rhodesiense**. It must be administered intravenously often with numerous toxic side effects. This leaves no safe drug available for treatment of trypomastigote stages in the blood of humans. Once trypomastigote stages cross the blood brain barrier the only compounds used are melarsorprol and nitrofurazone. Parasites resistant to melarsoprol are frequently found and toxicity is a severe problem. Nitrofurazone has numerous toxic side effects leaving no reliable therapeutic treatment for human cerebral African trypanosomiasis.

Many new compounds are cross resistant with parasites resistant to both pentamidine and melarsoprol. Therefore new compounds must be tested for degree for cross resistance patterns to established antitrypanosomal compounds.

The resistance of **T. rhodesiense** to selected antitrypanosomal compounds can be induced by repeated drug pressure in an **in vivo** test system. This was achieved by infecting mice with a standard inoculum of parasites, administering the test compound at a dose just below the curative level, and passing parasites from these animals to a new set of mice when the parasitemia rose to a desirable level. Passes were made every 3 to 4 days with drug doses being increased as resistance develops at each dose level.

This type of study can establish the rate at which **T. rhodesiense** acquires resistance in mice to selected compounds. Degrees of cross resistance of trypanosomicidal compounds found to be active against the drug-resistante lines may also be determined.

Lines of trypanosomes have been developed which are completely resistant to the following compounds.

RESISTANT LINES	HIGHEST DOSE RESISTANCE ACHIEVED
Pentamidine	212.0 mg/kg
Melarsoprol (Mel-B)	424.0 mg/kg
Suramin	543.0 mg/kg

METHODS

ANIMALS HOSTS

CD-1 Swiss mice (**Mus musculus**) used in this screening procedure weighed 20 to 24 grams with weight variations in any given experimental or control group carefully limited to 3 grams. Both male and female mice were used and were approximately the same age.

Animals were housed in metal-topped plastic cages, fed a standard laboratory diet and given water **ad libitum**. After drug treatment, mice were kept in a room maintained at a temperature of 28.8° (±2°C) with a relative humidity of 66% (±2%).

INOCULATION OF PARASITES

Giemsa-stained blood smears from donor mice infected 3 days earlier with **T. rhodesiense** trypomastigotes were microscopically examined to determine parasitemias (i.,e., number of trypomastigotes in a field of 100 erythrocytes). One set of test animals was infected with the drug-sensitive line of parasites by receiving an intraperitoneal injection of 0.2 cc of a $1:1.5X10^4$ dilution of heparinized heart blood drawn from a donor mouse harboring a parasitemia of 30-35% (approx. $1.3X10^4$ - $1.7X10^4$ trypomastigotes). Other sets of mice were similarly infected with each drug-resistant line to be tested. Blood dilutions were made such that all mice infected with the resistant lines received approximately the same number of trypomastigotes as mice infected with the drug-sensitive line.

Groups of 10 mice per group infected with the drug-sensitive line and with each drug-resistant line but receiving no drug served as negative controls.

DRUG ADMINISTRATION

Test compounds were dissolved or suspended in either peanut oil for subcutaneous administration or HEC for oral administration. Compounds were given 1 hour following challenge with trypomastigotes.

Compounds doses were diluted 2 or 4-fold from a level that had been projected to be fully curative. Five mice were used for each dose level.

CROSS RESISTANCE DETERMINATION

Each compound was tested against the drug-sensitive line and the 3 drug-resistant lines. Mice surviving 30 days postinfection were considered cured. The degree of cross resistance (fold resistant) was obtained by the following calculation.

$$\text{Cross-resistance (Fold resistant)} = \frac{CD_{50}\ \text{Drug-resistant line}}{CD_{50}\ \text{Drug-sensitive line}}$$

CD_{50} is the lowest mg/kg level of a compound curing at least 3 of 5 mice.

RESULTS

EXPERIMENTAL DATA

Infected non-treated control mice for all lines of trypanosomes died on either day 4 or 5 postinfection.

There were 91 compounds tested against the drug-sensitive line and the 3 lines resistant to either melarsoprol, suramin and pentamidine. The 26 compounds exhibiting no cross resistance with either of the 3 resistant lines are listed below.

BH 89189	BJ 34410	BK 03170
AM 37314	BJ 39273	BK 03205
BK 23976	BJ 39282	BK 15321
BK 51970	BJ 44783	BK 63596
BJ 45271	BK 65367	AX 37252
BK 62820	BG 81624	AP 76740
BH 03081	BK 65349	BJ 01377
BJ 93599	BK 11770	BK 03116
BK 15330	BK 03198	

One compound was resistant to only melarsoprol and its bottle number is a BK 51890. Six compounds were resistant to only suramin and their bottle numbers are

BH 89189	ZC 68487	AK 27774
BJ 33664	BH 05629	BK 15607

Four compounds were resistant to only pentamidine and their bottle numbers are BK 63194, AB 45932, BG 81615, AND BJ 42510.

CONCLUSIONS

In the primary antimalarial test system (MM) 167 of 1502 compounds tested exhibited asexual blood schizonticidal activity. This testing needs to be continued in order to find new chemical classes active against malaria and also to evaluate compounds emerging out of the lead directed synthesis programs.

Selected active compounds previously tested in the MM test were examined in a greater detail a modified MM test. Arteether in sesame oil more active SC than PO. Two of 3 artemisinin analogs (BL 55811 and BL 55795) were active when administered on D+3, 4 and 5 while BL 55802 was inactive. Sodium artelinate was active when administered PO at 80 mg/kg/day for 3 days. Mefloquine enhanced the curative activity of sodium artelinate when they were administered simultaneously. One of 2 trioxanes (BL 52276) had suppressive activity both SC and PO when administered for 3 days. The other trioxane (BL 52285) was active only SC. Chloroquine was less toxic when administered SC in SuperMax EPA oil and PO in a water soluble vitamin E solution. Primaquine was less toxic SC in SuperMax EPA oil.

One sustained release formulation of qinghaosu (BL 47408) was more active than another (BL 47417).

Two stereoisomers of a floxacrine analog interacted synergistically together against malaria. Mefloquine potentiated the antimalarial activity of Na artelinate.

Desiparmine did not reverse chloroquine resistance in a P. yoelii line containing a small population of highly chloroquine-resistant parasites.

Supplemental vitamin E did not alter the antimalarial activity of primaquine, chloroquine, qinghaosu or Na artelinate. Various plant and fish oils did administered to infected mice did not alter the course of infection. Other chemicals such as $FeSO_4$, $CuSO_4$, malonaldehyde or alloxan did not alter malarial infections.

Fifteen experiments were done to study if malarial infections could be controlled by changing the fatty acid profiles of red blood cell membranes in conjunction with lowering the vitamin E level in mice. The oil component in the diet of mice was changed to various fish and plant oils containing increased levels of omega-3 polyunsaturated fatty acids. Chloroquine resistant and qinghaosu resistant parasites were also susceptible as drug-sensitive parasites to this change in membrane fatty acid changes. Paraaminobenzoic acid levels were also shown to influence the level of malaria parasite growth.

Resistance to the R-stereoisomer of a floxacrine analog developed slower than the S-stereoisomer or the parent floxacrine analog itself. A line resistant to qinghaosu was developed.

There were 360 compounds evaluated for activity against drug-sensitive **Trypanosoma rhodesiense**. A total of 55 these were active. Testing of 91 active compounds was done against three different drug-resistant lines. These lines were resistant to either melarsoprol, pentamidine or suramin. Twenty-six compounds were not cross resistant to either of the three resistant lines. Eleven compounds were resistant to only one of the three resistant lines.

ACKNOWLEDGMENENT

The personnel at the **CENTER FOR TROPICAL PARASITIC DISEASES** participating in this Chemotherapy of Malaria project deserve a tremendous degree of credit for an excellent performance.

CHEMOTHERAPY ASPECTS

Joaquin Ardavin

Merida Aviles

Rosa Fontela

Richard May

CARE AND MAINTENANCE OF ANIMAL COLONY

Ian Squires

Phillip Roberts

MAINTENANCE OF LABORATORY COMPLEX

Armando Redondo

STAFF ASSOCIATE

Maria Isabel Ager

PRINCIPAL INVESTIGATOR

DR. ARBA L. AGER, JR.

DISTRIBUTION LIST

5 copies	Director Walter Reed Army Institute of Research Walter Reed Army Medical Center ATTN: SGRD-UWZ-C Washington, D.C. 20012
1 copy	Commander US Army Medical Research and Development Command ATTN: SGRD-RMI-S Fort Detrick, Frederick, MD 21702-5012
2 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-FDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Dean School of Medicine Unformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20824-4799
1 copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100